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Notch signaling in blood vessels: from morphogenesis to homeostasis

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Notch signaling is an evolutionarily conserved intercellular signaling pathway that plays numerous crucial roles in vascular development and physiology. Compelling evidence indicates that Notch signaling is vital for vascular morphogenesis including arterial and venous differentiation and endothelial tip and stalk cell specification during sprouting angiogenesis and also vessel maturation featured by mural cell differentiation and recruitment. Notch signaling is also required for vascular homeostasis in adults by keeping quiescent phalanx cells from re-entering cell cycle and by modulating the behavior of endothelial progenitor cells. We will summarize recent advances of Notch pathway in vascular biology with special emphasis on the underlying molecular mechanisms.

Notch pathway, vascular morphogenesis, vascular homeostasis, endothelial cells, mural cells

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As the most widely distributed organ in the body, blood vessels supply oxygen and nutrients and bring away metabolic wastes. Blood vessels also support tissue growth and repair and provide gateways for immune surveillance. The abnormalities in vessel structure or function contribute to many diseases. Insufficient vessel growth or maintenance aggravates ischemic diseases such as myocardial infarction and stroke, whereas excessive vascularization or abnormal remodeling promotes many diseases including cancer, metabolism-associated disorders, inflammation, and eye-related degenerative diseases [1–3].

In early embryonic and postnatal stages, the highly organized and functionally competent vascular networks are progressively assembled essentially through vasculogenesis and angiogenesis. Vasculogenesis refers to the process of forming major embryonic vessels by coalescence of individual endothelial progenitor cells (EPCs) or angioblasts that arise *de novo* from extraembryonic and embryonic lateral mesoderms. These progenitors form vesicles and cords of attached vascular endothelial cells (ECs) that undergo further morphogenesis to form endothelial tubes. Angiogenesis is defined as the formation of new vessels from preexisting vessels, either by sprouting and elongation of new vessels from the existing vessels (sprouting angiogenesis) or by remodeling of vessels via internal division of preexisting vessels in the capillary plexus (intussusceptive angiogenesis). Most blood vessel formation in later developmental and postnatal stages occurs via angiogenesis, which is halted in most adult tissues due to mature ECs staying in quiescence to maintain vascular homeostasis, except for the ovarian and the uterine epithelium during the menstrual cycle. In response to angiogenic signals, not only a part of mature ECs regain the ability of angiogenic prolif-

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eration and differentiation, but also EPCs preserved in the bone marrow (BM) are mobilized to contribute to postnatal vasculogenesis, leading to neovascularization in adults [2,4].

Since the early discoveries in late 1990s demonstrating the essential roles of Notch signaling in physiological vasculature and cardiovascular disorders in Alagille syndrome, a Notch pathway-wired signaling network in vessel development and function has emerged based on a large array of evidence from both experimental and genetic studies [5–7]. Recently, critical signaling knots and connections with other pathways of this network have been depicted. Moreover, increasing data have shown that the Notch signaling pathway also plays a pivotal role in vascular homeostasis and remodeling. In this review, we will discuss the physiological roles of Notch pathway in vascular development, providing an overview on the involvement of Notch signaling in vascular biology ranging from morphogenesis to homeostasis in the adult.

1 The core Notch signaling pathway

Notch signaling is an evolutionarily highly conserved pathway mediating cell contact-dependent signaling between neighboring cells in metazoans [8]. Signaling mediated by Notch receptors and ligands is involved in the regulation of many developmental events, such as cell differentiation, proliferation and apoptosis during embryogenesis and homeostasis of adult self-renewing tissues [8]. Notch signal may specify cell fates via lateral inhibition or induction, in which a cell takes a fate different from its signal-sending neighboring cells or is instructed to a specific fate, respectively [8,9]. In mammals, there are five Notch ligands (Jagged (Jag) 1 and 2 and delta-like (Dll) 1, 3 and 4) and four Notch receptors (Notch1–4), both of which are type I transmembrane proteins. Notch receptors are activated by binding to the ligands, which triggers two steps of sequential receptor cleavages within the transmembrane domain catalyzed by ADAM17 and γ-secretase, respectively. These cleavages result in the release of the Notch intracellular domain (NICD) into the cytoplasm. NICD then translocates into the nucleus and binds to the transcription factor CSL (C promoter-binding factor 1 (CBF-1)/suppressor of hairless (Su(H))/Lin-12 and Glp-1 (LAG-1), also known as recombination signal-binding protein-J kappa (RBP-J) in rodents). Association of NICD and CSL replaces a corepressor complex from CSL with a coactivating complex containing the Mastermind-like (MAML) protein, and activates the transcription of downstream genes such as the Hes (Hairy and enhancer of split) family molecules Hey1, Hey2 [7]. Recently several groups have utilized CHIP (chromatin immunoprecipitation) array to identify downstream effectors of Notch pathway [10–12], discovering hundreds of direct downstream candidate genes of canonical Notch signaling. In addition, NICD has been shown to interact with many other signal transduction molecules to regulate cell behavior independent on CSL factors, constituting the non-canonical Notch signaling [10].

2 Notch signaling in vessel morphogenesis

2.1 Vasculogenesis

The development of the vascular system begins with the formation of hemangioblasts, which are organized into blood islands in the yolk sac. Hemangioblasts at the outer region of blood islands form angioblastic cells, and aggregate into primitive vessels after migration. Primitive vessels need to differentiate into a hierarchically organized network of arteries, capillaries, and veins. Arteries are supported by layers of vascular smooth muscle cells (vSMCs) and a specialized matrix, and are characterized by higher expression of Ephrin-B2. Veins are thinner and surrounded by fewer vSMCs, and are featured by expressing EphB4, the ligand for Ephrin-B2 [1,13,14]. The initial expression of markers of arterial and venous identity occurs before the initiation of heart beat and circulatory flow, suggesting that genetic determinants rather than hemodynamic factors such as blood flow and pressure play a critical role in instructing the initial steps of arterial/venous fate determination [1]. Now it is clear that a variety of factors such as Notch, Hedgehog and Coup-TFII work in concert to specify arterial or venous fate [14].

Notch pathway components are highly expressed in arterial ECs but are low in veins. Intervention of Notch signaling in Dll4 heterozygous mice, endothelial-specific Hes1 and Hes5 mutant or Hey1 and Hey2 double-mutants all leads to loss of arterial markers and re-expression of venous signature genes in arterial ECs [15–18]. Overexpression of activated Notch4 in adult mice induced EphrinB2 expression in the venous compartment. These mice were characterized by arteriovenous malformation that was reversible after repression of Notch4 expression [19,20]. Recently, using transgenic zebrafish bearing a Notch reporter, Quillien et al. [21] have demonstrated that Notch signal is activated in endothelial progenitors during vasculogenesis prior to blood vessel morphogenesis, and that endothelial progenitors in which Notch signal is activated are committed to a dorsal aorta fate. Lineage analysis, together with perturbation of both Notch receptor and ligand function, further suggests several distinct developmental windows in which Notch signaling acts to promote artery commitment and maintenance. In response to Notch signaling, Ephrin-B2 expression increases in arterial ECs, whereas its receptor EphB4 in venous ECs is repressed by Notch signal [13]. Together, these findings demonstrate that Notch acts in distinct contexts to initiate and maintain artery identity during embryogenesis, partially by controlling the expression of Eph-Ephrin family members, which define arterio-venous

boundaries.

Notch signaling is also implicated in the regulation of EPCs in postnatal neovascularization [22–24]. Jagged1 and Dll1 are expressed by BM stromal niche cells and the reciprocal interaction between Jagged1, but not Dll1, and Notch receptors on EPCs stimulates EPC commitment, differentiation and mobilization [25]. Recently Ii et al. [26] have shown that Notch1 regulates EPC function and viability during recovery from arterial injury in hypercholesterolemic mice. With a mouse partial hepatectomy (PHx) model and RBP-J deficient mice, our group has shown that Notch-RBP-J signaling regulates the mobilization and function of endothelial progenitor cells by dynamic modulation of CXCR4 expression [27]. Further, we found that Notch signal might exert different roles in heterogenous EPC subsets [28]. In a choroidal neovascularization model, RBP-J deficiency induced a more intensive angiogenic response to injury. This could be rescued by BM transplantation, indicating that RBP-J could modulate BM-derived EPCs in adult vascularization [29]. These findings are likely to lead to the advancement of cell-based therapies for vascular regeneration for ischemic vascular diseases in the future.

2.2 Sprouting angiogenesis

Sprouting angiogenesis involves coordinative efforts of two distinct EC types, namely tip and stalk cells. Tip cells protrude filopodia extending to the vessel frontier and the following stalk cells proliferate to form the trunk of the new vessel sprouts [6,30]. Notch signaling plays a fundamental role in tip and stalk cell specification. The dynamic spatial and temporal expression of Dll4 helps to set up a “salt and pepper” pattern of tip and stalk cell distribution [30]. In response to VEGF stimulation, cells that have higher VEGFR2 signal upregulate Dll4 and adopt a tip cell fate to initiate sprouting [31]. Dll4 then activates the Notch pathway in adjacent ECs to reduce its expression of VEGFR2 and VEGFR3, thereby suppressing the tip cell phenotype and alternatively promoting the stalk cell phenotype [30,31]. In the absence of Notch signaling, ECs continue to form sprouts in response to VEGF, resulting in more sprouts and branches per sprout, as has been documented in different model systems such as zebrafish embryos [32], mouse retina [33–35] and xenograft tumors [36–38]. More recently, a role of Notch signaling has also been demonstrated in decidual angiogenesis and luteal angiogenesis [39]. Jagged1, another type of Notch ligands, plays a proangiogenic role in mice by antagonizng Dll4-Notch signaling in cells expressing Fringe family glycosyltransferases (Figure 1) [34].

2.3 Vessel maturation

A fundamental feature of vessel maturation is the recruitment of mural cells, which consist of vSMCs and pericytes. Lineage mapping studies have identified vSMCs derived from a variety of embryonic sources such as neuronal crest, pericardium, mesothelium, secondary heart field, somites, and mesoangioblasts. Recently Chang et al. have displayed that a local $Tie1^{+}$ CD31dimVEcad-$CD45^{-}$ precursor can differentiate into vSMCs in all vascular beds and this process requires Notch activation [40]. Even committed skeletal myoblasts can be converted into functional pericytes via Dll4 and PDGF-BB [41].

Notch signals on vessel endothelial cells also control maturation and arterial differentiation of vSMCs [42]. Endothelial-specific Jagged1 mutants have impaired vSMC differentiation and valve morphogenesis defects in both embryonic and adult mice, reminiscent of Alagille syndrome [43,44]. While Jagged1 is the major ligand important for this process, Notch3 seems to be the critical receptor for mural cell differentiation. Notch3 is predominantly ex-

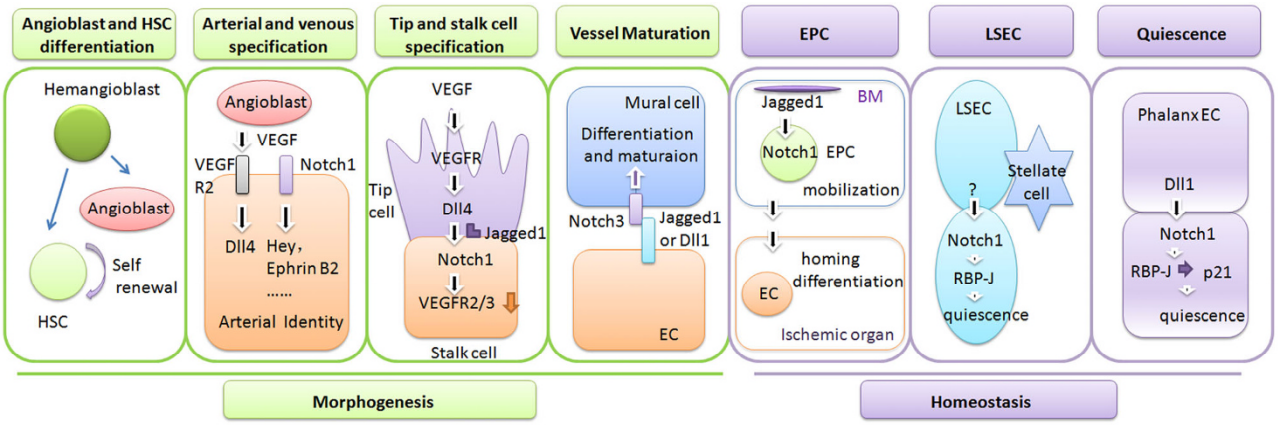

Figure 1 (color online) Notch pathway plays various roles in vessel morphogenesis and homeostasis. Notch pathway is dispensable for hemangioblast differentiation into angioblast, while is required for adult HSC self-renewal. In arterial and venous differentiation, Notch signal is fundamental for arterial identity and maintenance. The complex interaction between Notch and VEGFR signaling is crucial for tip and stalk cell specification. Notch signal keeps on working by regulating mural cell differentiation and recruitment in vessel maturation in later stages of vessel morphogenesis. Furthermore, Notch signaling pathway is required for adult vessel homeostasis by modulation of EPC differentiation and mobilization and maintenance of EC quiescence through p21.

pressed in arterial vSMCs and is not expressed in veins. Notch3 deletion impairs mural cell investment, resulting in progressive loss of vessel coverage [45,46], whereas Notch3 mutations in humans cause degeneration of vSMCs in CADASIL, a human stroke and dementia syndrome [47–50]. Up-regulation of PDGFRβ expression by Notch signal may contribute to Notch3-regulated mural cell investment, as demonstrated by Jin et al. [51]. Scheppke et al. [52] further documented that Jagged1 on ECs promotes the expression of integrin αvβ3, which facilitates vSMCs adhesion to endothelial basement membrane and vessel maturation. These observations indicate that Jagged1 expression by ECs activates Notch signaling in neighboring cells and drives vSMC differentiation and maturation [53,54], possibly by inducing the expression of PDGFRβ and integrin αvβ3.

A very recent study has demonstrated that Notch/VEGFR-regulated differential dynamics of VE-cadherin junctions drive functional EC rearrangements during sprouting [55]. Continual flux in Notch signaling levels in individual cells results in differential VE-cadherin turnover and junctional-cortex protrusions, which drive differential cell movement. In cultured ECs, Notch signaling quantitatively reduces junctional VE-cadherin mobility. Only differential adhesion dynamics generates long-range position changes, which is required for tip cell competition and stalk cell intercalation [55]. Computational simulation and quantitative image analysis of VE-cadherin junctional patterning in vivo have identified that differential VE-cadherin mobility is lost under pathological high VEGF conditions, such as retinopathy and tumour vasculature [55]. These results provide a mechanistic explanation for how cells rearrange during normal sprouting and how rearrangement switches to generate abnormal vessels in pathologenesis.

2.4 Vessel remodeling and regression

The initial endothelial plexus created by vascular sprouting is a homogenous EC network, and is generated in excess. The final adjustment of vascular density involves the regression of unnecessary vessels through vascular remodeling and pruning. Blood flow is critically important for vessel remodeling and regression by modulating the expression of shear stress-responsive transcription factor Kruppel-like factor 2 (KLF2), which promotes EC quiescence and the formation of stable vessels with an anti-thrombogenic endothelial lining, while hypoperfused vessels with low KLF2 expression may undergo apoptosis or regression instead [30]. Notch signaling also plays a role in vessel remodeling and regression. Inhibition of Notch signaling prevented vessel regression both in normal retinal development and in the oxygen-induced retinopathy model in mice [56]. Interestingly, Dll4/Notch inhibition lead to increased expression of the vasodilator adrenomedullin and suppressed expression of the vasoconstrictor angiotensinogen in a VEGF-independent manner [56]. In addition, recent data have also demonstrated that Notch signaling in macrophages is important for their localization and interaction with ECs in vessel branch fusion during sprouting angiogenesis [57].

3 Notch signaling in vessel homeostasis

The homeostasis of the vascular structure in normal adults is critical for the structure and function of organ parenchyma. Neovascularization in the adult retina, for instance, acts as a pivotal pathological process contributing to the age-related macular degeneration or diabetic retinopathy. Understanding the mechanisms governing vascular homeostasis and ECs quiescence in adult organs thus has important clinical implications [7]. Using the Cre-LoxP-mediated conditional gene deletion, our group demonstrated in adult mice that disruption of RBP-J strikingly induced spontaneous angiogenesis in multiple tissues, including retina and cornea, as well as in internal organs, such as liver and lung [29]. Accumulative vessel outgrowth may result from significantly increased proliferation of ECs, possibly through upregulation of VEGFR2 expression in RBP-J deficient ECs [29]. In addition, ECs in RBP-J deficient mice exhibit a significant down-regulation of VEGFR1, which is responsible for maintaining the corneal avascularity by antagonizing proangiogenic factors [29]. These findings suggest that RBP-J-mediated Notch signaling may play an essential role in the maintenance of vascular homeostasis in adults by repressing EC proliferation.

Liver sinusoid ECs (LSECs) are specialized ECs lining the surface of LSs, which function to filter serum, regulate hepatic microcirculation, and participate in metabolism. LSECs have complicated communications with hepatocytes, and play critical roles in supporting liver development and regeneration [58]. LSECs also participate in the pathogenesis of many liver diseases [59], such as the BM transplantation-induced veno-occlusive disease, which is characterized by congested LSs, fibrin deposition, subendothelial edema, and hepatocellular abnormalities. Conditional deletion of RBP-J in adult mice resulted in LSEC proliferation and a veno-occlusive disease-like phenotype in the liver [60]. Regeneration of liver after partial hepatectomy (PHx) was remarkably impaired, with reduced LSEC proliferation and destroyed sinusoidal structure [60]. These results indicated that Notch/RBP-J signaling may play dual roles in LSECs: in resting liver it represses proliferation, and in regenerating liver it supports proliferation and function of LSECs.

4 Prospective

Although we have witnessed great advances in uncovering the critical roles of Notch pathway in vascular development and homeostasis in the past decades [7,61], the current low-resolution picture of Notch function in vasculature pro-

vided limited mechanistic and clinical-relevant insights, raising many important open questions for future studies.

(i) The Notch pathway cross-talks with numerous other pathways and molecules through canonical and non-canonical signaling. Molecular interactions between Notch and other pathways should be identified to depict the molecular network governing vascular morphogenesis and homeostasis. For instance, it has been shown recently that critical metabolism molecules and pathways are involved in vessel sprouting [62–64]. Moreover, cell adhesive molecules appear to play more and more important roles in vascular development [55,65,66]. It will be interesting to elucidate whether Notch signaling interacts with these pathways to modulate vessel morphogenesis and homeostasis. Identification of downstream molecules is another important task to explain the mechanism of Notch signaling in vessels. Although multiple epigenetic mechanisms have been revealed in vessel development and homeostasis [67,68], their relationship with Notch signaling is still obscure.

(ii) To what extent does Notch signaling regulate endothelial functions? Dll4 induces Notch signaling in macrophages and participates in inflammation [69]. We and others have shown that Notch signaling participates in oxidative responses in both ECs and tissue parenchymal cells [70,71]. Moreover, it is noteworthy that Notch signaling in ECs and vSMCs regulates vasoreactivity of blood vessels [56]. Analyses of different Notch reporter mice and gene modified mice of different Notch pathway components, as well as comprehensive bioinformatic integration, will be helpful to dissect the exact role of this pathway in endothelial functions [72].

(iii) Notch signaling in vessel-related diseases will be another research hot-point in this field. Genetic and animal model studies have identified mutations of Notch signaling as etiological reasons of a few human diseases, including Cadasil syndrome [73], Alagile syndrome [74], familial tetralogy of Fallot [75], and so on. Notch activation is also implicated in EC senescence and pro-inflammatory response [76], which might involve the SIRT1 deacetylase [77]. Dll4 blockade attenuates atherosclerosis and metabolic disorders [78]. Furthermore, given the central roles of Notch in the development and homeostasis of vascular morphogenesis and homeostasis, Notch is long regarded as a highly desirable therapeutic target from a translational perspective [79]. We have invented an EC-targeted soluble human Dll1 and evaluated its effects on suppressing tumor angiogenesis [80]. A more comprehensive understanding of Notch signal in vascular biology may lead to better therapeutic strategies to control angiogenesis in relevant diseases.

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